INVENTION TITLE

GALENIC FORM FOR COLON-TARGETED DELIVERY
OF ACTIVE INGREDIENTS

DESCRIPTION

[Para 1]   The present invention relates to a galenic form for colon-targeted delivery, its preparation process and its use in therapeutic.

[Para 2]   Specific release systems in the colon have been proven to have significant theracanic advantages.

[Para 3]   A large number of colonic illnesses could effectively be treated more efficaciously if the active ingredient were released locally. This is the case, inter alia, for Crohn's disease, ulcerative colitis, colorectal cancer and constipation.

[Para 4]   Colon-targeted release could also be interesting when, from the theracanic point of view, a delay in absorption is necessary, in particular in the treatment of pathologies such as nocturnal asthma or angor (Kinget R. et al. (1998), Colonic Drug Targeting, Journal of Drug Targeting, 6,129).

[Para 5]   The administration of polypeptidic active ingredients occurs essentially parenterally, which is painful and the origin of poor observation of treatment. For some years now there has been an interest in using the colon as an absorption site for peptidic active ingredients (analgesic, contraceptive, vaccine, insulin...). The absorption of peptides in the colon seems effectively better than at other sites of the digestive tract, in particular due to proteolytic activity clearly weaker than in the small intestine and in the absence of peptidasic activity associated with the membrane of the epithelial colonic cells.

[Para 6]   During oral administration of antibiotics, these pass through the stomach and are then absorbed in the small intestine to diffuse in the entire organism and treat the infectious focus for which they have been administered. All the same, a fraction of ingested antibiotics (whereof the importance varies with the characteristics peculiar to each type of antibiotic) is not absorbed and continues its progress to the colon before being eliminated in the stool. These residual antibiotics are reunited, in the small

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intestine, by a fraction of the antibiotics absorbed, but which are re-excreted in the digestive tract by way of biliary elimination. This fraction is of variable importance as a function of metabolism and elimination paths of each antibiotic. Finally, for certain antibiotics, a fraction of the dose absorbed is eliminated directly by the intestinal mucous in the lumen of the digestive tract. Since the antibiotics had been administered orally or parenterally, an active residual fraction is generally found in the colon. This holds true, to varying degrees, for the vast majority of the families of antibiotics utilised in therapeutics, the only notable exception being the family of amino-glycosides for which intestinal excretion is negligible. For the other antibiotics, intestinal excretion of a residual antibiotic activity will have different consequences, all harmful. In effect, in the colon there is a complex (several hundreds of different bacterial species) and very dense (more than 10^{11} bacteria per gram of colonic content) bacterial ecosystem which will be affected by the arrival of active residues of antibiotics. The following is observed:

1) imbalance of flora which would be the main cause of banal diarrhoea sometimes following ingestion of antibiotics (Bartlett J. G. (2002) Clinical practice. Antibiotic associated diarrhea, New England Journal of Medicine, 346, 334). Even though this diarrhoea is generally not serious and quickly abates, either spontaneously, or when treatment is discontinued, it is nevertheless badly received by patients and adds to the discomfort of the basic illness for which antibiotics have been prescribed;

2) perturbation of the functions of resistance to colonisation by exogenic bacteria (or " barrier effect ") with the possibility of increased risk of infection, for example, alimentary intoxication to Salmonella (Holmberg S. D. et al. (1984) Drug resistant Salmonella from animals fed antimicrobials, New England Journal of Medicine, 311, 617);

3) selection of micro-organisms resistant to antibiotics. The latter can be of various types:

a) they can first be pathogenic bacteria, such as for example Clostridium difficile, a species capable of secreting toxins causing redoubtable colitis known as pseudomembranous (Bartlett J. G. (1997) Clostridium difficile infection: pathophysiology and diagnosis, Seminar in Gastrointestinal Disease, 8,12);

b) they can also be relatively slightly pathogenic micro-organisms but whereof the multiplication can lead to surrounding infection (vaginal Candidosis or Escherichia coli resistant cystitis).

c) they can finally be non-pathogenic commensal resistant bacteria but

whereof the multiplication and faecal elimination will boost dissemination in the environment. As it is, these resistant commensal bacteria can constitute an important source of resistance mechanisms for pathogenic species. This risk is currently considered to be major in terms of the worrying character in the evolution to multiresistance of numerous pathogenic species for humans.

[Para 7]   Numerous strategies exploiting the diverse physiological parameters of the digestive tract have therefore been envisaged with a view to releasing active ingredients in the colon. Studies have in particular been carried out by means of administration systems based on (1) utilisation of polymers sensitive to variations in pH, (2) release forms dependent on time, (3) prodrugs or again polymers degradable by bacteria in the flora.

(1) Systems based on variations in pH.

[Para 8]   The pH in the stomach is of the order of 1 to 3 but it increases in the small intestine and colon to reach values close to 7 (Hovgaard L. et al. (1996) Current Applications of Polysaccharides in Colon Targeting, Critical Reviews in Theracanic Drug Carrier Systems, 13,185). For an active ingredient to reach the colon, without undergoing these variations in pH, it is possible to administer it in the form of tablets, gels or spheroids coated in a pH-dependent polymer, insoluble in acid pH but soluble in neutral or alkaline pH (Kinget et al. op cit). The most commonly used polymers are derivatives of methacrylic acid, Eudragite L and S (Ashford M. et al. (1993), An in vivo investigation into the suitability of pH-dependent polymers for colonic targeting, International Journal of Pharmaceutics, 95,193 and 95,241; and David A. et al. (1997) Acrylic polymers for colon-specific drug delivery, S. T. P. Pharma Sciences, 7, 546).

[Para 9]   Given the important inter- and intra-individual variability of the values of pH at the gastro-intestinal tract level, pH-dependent polymers do not represent the best means for obtaining specific release in the colon (Ashford M. et al., op cit.).

(2) Systems based on transit time.

[Para 1 0]   The formulation of these systems is such that it allows release of the active ingredients after a predefined lag time. So as to release the active ingredient in the colon, these forms must still resist the acid environment of the stomach and enter a silent

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phase of a predetermined time, before releasing the active ingredient, corresponding to the transit time from the mouth to the terminal ileon (Gazzaniga A. et al. (1995) Time-dependent oral delivery systems for colon targeting, S.T. P. Pharma Sciences, 5,83 and 108, 77; Liu P. et al. (1999) Alginate/Pectin/Poly-L-lysine particulate as a potential controlled release formulation, J. Pharm. Pharmacol., 51, 141; Pozzi F. et al. (1994) The Time Clock system: a new oral dosage form for fast and complete release of drug after predetermined lag time, Journal of Controlled Release, 31,99).

[Para 11] Pulsincap® by Scherer was one of the first formulations of this type (international patent application WO90/09168). It has the appearance of a gel whereof the body is insoluble in water. The active ingredient is maintained in the body by a hydrogel stopper placed in the head of the hydrosoluble gel. The whole is coated in a gastro-resistant film. After dissolution of the head in the small intestine, the stopper swells on contact with digestive juices. When the latter reaches a critical swelling threshold it is ejected, thus allowing release of the active ingredient. The ejection time is controlled by the properties of the hydrogel constituting the stopper.

[Para 12] Systems based on transit time nevertheless offer numerous disadvantages (variations in time for emptying of stomach and transit time, retention phenomena in the ileo-caecal valve (Kinget R., op. cit.), causing a lack in specificity and preventing validation of the latter as specific release systems in the colon. Finally, large-scale production of this type of system is difficult to envisage, as this would require and costly significant adaptation of industrial technologies.

[Para 13] Recently a novel form for colonic targeting has been developed, "Colon-targeted Delivery Capsule" (CTDC) (Ishibashi T. et al. (1998) Design and evaluation of a new capsule-type dosage form for colon-targeted delivery of drugs, International Journal of Pharmaceutics, 168,31 and 57,45). The CTDC is a system bringing together the pH-dependent factor and the time-dependent factor. It is in the form of a classic gel encasing the active ingredient and an organic acid (succinic acid), covered in 3 layers.

(3) Systems based on enzymatic activity of the microbial colonic flora.
3.1. Prodrugs.

[Para 14] Prodrugs have largely been studied for colonic targeting of various active ingredients (anti-inflammatory non-steroidal and steroidal, spasmolytic, ...). These systems are based on the capacity of the enzymes produced by the colonic flora for degrading prodrugs in order to release the active form of the active ingredient.

[Para 15] Numerous prodrugs based on the action of the bacterial azoreductases in particular have been developed with the aim of releasing in the colon active ingredients such as 5-aminosalicylic acid (5-ASA) utilised in the treatment of local pathologies such as Crohn's disease or ulcerative colitis (Peppercorn M. A. et al. (1972) The role of intestinal bacteria in the metabolism of salicylazosulfapyridine, The Journal of Pharmacology and Experimental Therapeutics, 181, 555 and 64, 240).

[Para 16] Another approach consists of exploiting bacterial hydrolases such as glycosidases and polysaccharidases (Friend D. R. (1995) Glycoside prodrugs: novel pharmacotherapy for colonic diseases, S. T. P. Pharma Sciences, 5, 70 Friend D. R. et al. (1984) A colon-specific drug-delivery system based on drug glycosides and the glycosidases of colonic bacteria, Journal of Medicinal Chemistry, 27, 261; Friend D. R. et al. (1985) Drug glycosides: potential prodrugs for colon-specific drug delivery, Journal of Medicinal Chemistry, 28, 51; and Friend D. R. et al. (1992) Drug glycosides in oral colon-specific drug delivery, Journal of Controlled Release, 19, 109). Prodrugs have thus been developed by coupling, for example, steroids to sugars (glucose, galactose, cellobiose, dextran (international patent application WO90/09168)), cyclodextrins (Hirayama F. et al. (1996) In vitro evaluation of Biphenylyl Acetic Acid-p-Cyclodextrin conjugates as colon-targeting prodrugs: drug release behavior in rat biological media, Journal of Pharmacy and Pharmacology, 48, 27).

3.2. Coating by polymers biodegradable by bacterial enzymes.

[Para 17] In this case, colonic targeting is done by coating the pharmaceutical form with a polymer specifically degraded by the enzymes produced by microflora, by benefiting from the presence of azoreductases or bacterial glycosidases.

[Para 18] Numerous polymers including azoaromatic links have been used to coat an active ingredient. Saffran et al. (Oral insulin in diabetic dogs, Journal of Endocrinology (1991), 131, 267 and A new approach to the oral administration of insulin

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and other peptide drugs, Science (1986), 233, 1081) have described the release of insulin and vasopressin in the colon of rats and dogs from oral forms coated with copolymers of styrene and hydroxyethylmethacrylate (HEMA) linked by azoaromatic bonds. This coating is degraded in the colon by bacterial azoreductases responsible for release of the active substance.

[Para 19] The advantage of azopolymers is that they allow very good colonic selectivity for release of active ingredients. The disadvantage associated with use is the lack of information on their possible toxicity.

[Para 20] To avoid this disadvantage, other studies have chosen to focus on the use of coating film based on natural substance such as polysaccharides in particular with coating films based on amylose/ethylcellulose (Milojevic S. et al. (1996) Amylose as a coating for drug delivery to the colon: preparation and in vitro evaluation using 5-aminosalicylic acid pellets, Journal of Controlled Release, 38, 75), based on dextrane ester (Bauer K. H. et al. (1995) Novel pharmaceutical excipients for colon targeting, S. T. P. Pharma Sciences, 5, 54) or pectin.

3.3. Matrices biodegradable by bacterial enzymes.

[Para 21] Another approach of systems of specific release in the colon consists of the elaboration of matrices by compression of a mixture of active ingredient and biodegradable polymers such as chondroitin sulfate (Rubinstein A. et al. (1992b) Chondroitin sulfate: a potential biodegradable carrier for colon-specific drug delivery, International Journal of Pharmaceutics, 84, 141 and Rubinstein A. et al. (1992a) Colonic drug delivery: enhanced release of Indomethacin from cross-linked chondroitin matrix in rat cecal content, Pharmaceutical Research, 9,276), guar gum (Krishnaiah Y. S. R. et al. (1998) Evaluation of guar gum as a compression coat for drug targeting to colon, International Journal of Pharmaceutics, 171,137), chitosan (Tozaki H. et al. (1997) Chitosan capsules for colon-specific drug delivery: improvement of insulin absorption from the rat colon, Journal of Pharmaceutical Sciences, 86,1016) or pectin (Rubinstein A. et al. (1993) In vitro evaluation of calcium pectinate: a potential colon-specific drug delivery carrier, Pharmaceutical Research, 10, 258).

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[Para 22] Systems based on enzymatic activity of microbial flora are probably those having the greatest colonic specificity for release of the active ingredients. Therefore they make up a future path for colonic targeting.

[Para 23] The interest in polysaccharides in the preparation of systems for colonic administration is that they are of natural origin, only slightly toxic and specifically degraded by bacterial enzymes of the colonic flora.

[Para 24] Thus, pectin is a polysaccharide isolated from the cellular walls of superior vegetables, widely used in the agro-alimentary industry (as a gelling agent or thickener of jams, ices...) and pharmaceutical. It is polymolecular and polydisperse. Its composition varies according to the source, conditions of extraction and environmental factors.

[Para 25] Pectins are principally composed of linear chains of acids α-1,4- (D)-galacturonic, sometimes interspersed with units of rhamnose. The carboxylic groups of galacturonic acids can be partially esterified to give methylated pectins. Two types of pectin are distinguished according to their degree of methylation (DM: number of methoxy group per 100 units of galacturonic acid):

- highly methylated pectin (HM: high methoxy) whereof the degree of methylation varies between 50 and 80%. It is only slightly soluble in water and form gels in an acid medium (pH < 3.6) or in the presence of sugars;
- slightly methylated pectin (LM: low methoxy), with a degree of methylation from 25 to 50%. More soluble in water than HM pectin, it gives gels in the presence of divalent cations such as Ca^{2+} ions. In fact, Ca2+ ions form "bridges" between carboxylated groups free of galacturonic acids. The network thus formed has been described by Grant et al. Under the name of egg-box model (Grant G. T. et al. (1973) Biological interactions between polysaccharides and divalent cations: the egg-box model, FEBS Letters, 32, 195).

[Para 26] There are also amidated pectins. Certain groups of methyl carboxylate (-$COOCH_3$) can be transformed into carboxamide groups (-$CONH_2$) by treatment of pectin by ammonia. This amidation imparts novel properties to the pectins, in particular improved resistance to variations in pH.

[Para 27] The pectin is degraded by enzymes originating from superior vegetables and diverse micro-organisms (mushrooms, bacteria...) including bacteria of human colonic flora. The enzymes produced by the microflora are composed of polysaccharidases, glycosidases and esterases.

[Para 28] A galenic form is coated by pectin either via compression (Ashford M. et al. (1993b), An evaluation of pectin as a carrier for drug targeting to the colon, Journal of Controlled Release, 26, 213), or by pulverisation. Coating by compression is generally completed with pectin alone, whereas compression by pulverisation requires the use of a filmogenic polymer in addition to the pectin (Milojevic S. et al. (1996) Amylose as a coating for drug delivery to the colon: preparation and in vitro evaluation using 5-aminosalicylic acid pellets, Journal of Controlled Release, 38, 75; Wakerly Z. et al. (1996) Pectin/ethycellulose film coating formulations for colonic drug delivery, Pharmaceutical Research, 13, 1210).

[Para 29] Numerous matricial forms based on pectin have likewise been studied. They are generally constituted either by pure pectin, or by its complex with Ca^{2+} ions, slightly hydrosoluble, calcium pectinate. A matrix of calcium pectinate including indomethacin has in particular been described by Rubinstein et al. (1992a) Colonic drug delivery: enhanced release of Indomethacin from cross-linked chondroitin matrix in rat cecal content, Pharmaceutical Research, 9, 276) showing better stability of the calcium pectinate than the pectin alone in digestive juices, while remaining sensitive to the action of pectinolytic enzymes.

[Para 30] The amidated pectins, more tolerant to variations in pH have also been studied for elaboration of matricial tablets for colonic observation (Wakerly Z. et al. (1997) Studies on amidated pectins as potential carriers in colonic drug delivery, Journal of Pharmacy and Pharmacologyl. 49, 622).

[Para 31] Aydin et al.((1996) Preparation and evaluation of pectin beads, International Journal of Pharmaceutics, 137,133) were the first to formulate pectin beads according to the ionic gelification method by Bodmeier et al. ((1989) Preparation and evaluation of drug-containing chitosan beads, Drug Development and Industrial Pharmacy, 15, 1475 and Spherical agglomerates of water-insoluble drugs, Journal of Pharmaceutical Sciences, 78, 964), who had disclosed beads of alginate and chitosan.

Their objective was to incorporate in the beads two different active ingredients, a cationic (atenolol) and an anionic (piroxicam), so as to characterise possible interactions with pectin. They have thus demonstrated that it was possible to form beads with the 2 types of active ingredients and that the operational conditions had a major influence on the properties of the resulting beads.

[Para 32] Sriamornsak used beads of calcium pectinate to establish a system for specific release of proteins in the colon, by using bovine serum albumin (BSA) having a molecular weight of 66400 Da as protein model (Sriamornsak P. (1998) Investigation on pectin as a carrier for oral delivery of proteins using calcium pectinate gel beads, International Journal of Pharmaceutics, 169, 213 and (1999) Effect of calcium concentration, hardening agent and drying condition on release characteristics of oral proteins from calcium pectinate gel beads, European Journal of Pharmaceutical Sciences, 8, 221). He studied the influence of different factors of formulation on the properties of the resulting beads, such as their form, their size, the rate of encapsulation of the BSA and its release kinetics. Sriamornsak has therefore demonstrated that the pectinate beads of Ca could be employed for specific release of proteins in the colon. Obtaining an adequate release kinetic profile depends principally on the choice of the formulation and operational conditions for preparation of the beads. No in vitro/in vivo correlation of the release profiles of the encapsulated active ingredients has been established.

[Para 33] To boost the stability of the particles along the digestive tract and to avoid any premature release of the encapsulated active ingredient, it is possible to reinforce the pectin beads by reticulating them with a cationic polymer.

[Para 34] Munjeri et al.((1997) Hydrogel beads based on amidated pectins for colon-specific drug delivery: the role of chitosan in modifying drug release, Journal of Controlled Release, 46, 273) have reticulated pectin beads amidated with chitosan. They then showed, by comparing the kinetics of dissolution of reticulated forms and of non-reticulated forms, that the chitosan allowed the release of the active insoluble ingredients to be minimised, but did not significantly modify the release of the hydrosoluble active ingredients. The loss of active ingredient in conditions emulating those of the stomach and the small intestine can therefore be limited by formation of a complex between the chitosan and the amidated pectin; the reticulated pectin beads still remain sensitive to the action of the colonic pectinolytic enzymes.

[Para 35] Another reticulating agent, polylysine, has been tested in the presence of alginate/pectin beads (Liu P. et al. (1999) Alginate/Pectin/Poly-L-lysine particulate as a potential controlled release formulation, J. Pharm. Pharmacol., 51,141). The beads reticulated by the polylysine seem to release less active ingredient in an acid medium (HCl O,1N) than the non-reticulated beads, except in the presence of highly hydrosoluble active ingredients. The same type of effect is found in an alkaline medium (phosphate buffer, pH 7.5) but it is clearly less marked than in acid medium.

[Para 36] International patent application WO 88/07865 suggests administering bacteria producing β-lactamases in the colon so as to hydrolyse the residual antibiotics. The micro-organisms utilised are bacteria with strict anaerobic metabolism, whereof the production and lyophilisation in sufficient quantity to make a drug are difficult. Furthermore, they are carriers of genes resistant to the antibiotics encoding for β-lactamases thus engendering a risk of dissemination of these genes within the colonic ecosystem and in the environment.

[Para 37] International patent application WO 93/13795 proposes an oral galenic form containing β-lactamases. It can be composed of saccharose particles of 1 to 2,5 mm in diameter enclosing the β-lactamases or amidase and optionally an inhibitor of trypsine, said particles being covered by a gastroresistant polymer. These particles could well release the enzyme in different segments of the digestive tract so that its activity takes place as required at the desired site in the intestine.

[Para 38] None of the examples comprises experimental data showing that the proposed galenic formulation is effectively capable of releasing the enzyme in an active form at the desired site in the intestine. In addition, no proof of the capacity of galenic preparation for effectively hydrolysing the antibiotic in vivo, nor even in vitro in a medium reproducing the characteristics of the intestinal medium is given.

[Para 39] For all these reasons, it is highly desirable to use a system for reducing the quantity of residual antibiotics which reach the colon after oral or parenteral antibiotherapy, or capable of delivering an active ingredient directly to the colon.

[Para 40] Therefore, the object of the present invention is multiparticular galenic forms to be utilised orally and for colonic delivery of active ingredients.

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[Para 41] In the sense of the present invention, active ingredient is understood to mean a substance or composition which is suitable to be utilised in therapeutics or in diagnostics and can be incorporated in the galenic form according to the invention.

[Para 42] The active ingredient can be an anti-infectious, for example antibiotics, anti-inflammatory compounds, anti-histamines, anti-cholinergics, antivirals, antimitotics, peptides, proteins, genes, anti-sense oligonucleotides, diagnostic agents and/or immunosuppressive agents or bacteria.

[Para 43] Examples of particularly advantageous active ingredients are anti-inflammatory agents, antitumoral agents, anti-sense oligonucleotides and enzymes capable of inactivating antibiotics in the colon, in particular β-lactamases or enzymes capable of inactivating macrolids and related substances, such as erythromycin esterase described by Andremont A. et al.((1985) Plasmid mediated susceptibility to intestinal microbial antagonisms in Escherichia coli Infect. Immun. 49 (3), 751) or capable of inactivating quinolones such as those described by Chen Y et al.((1997) Microbicidal models of soil metabolisms biotransformations of danofloxacin, Journal of Industrial Microbiology and Biotechnology 19,378).

[Para 44] The active ingredients can be hydrosoluble or liposoluble.

[Para 45] In an advantageous embodiment of the invention, the multiparticular galenic forms suitable to be utilised orally and for colonic delivery of active ingredients comprise pectin beads in the form of a cationic salt enclosing the active ingredient, said pectin being reticulated by a cationic polymer.

[Para 46] In an advantageous embodiment according to the invention the cationic polymer which allows reticulation of the pectin is selected from the group composed of polyethylenimine, polylysine, chitosan and their derivatives.

[Para 47] More advantageously, the molecular weight of these cationic polymers, is between 10,000 and 100,000 Daltons, preferably between 20,000 and 50,000 Daltons.

[Para 48] In another advantageous embodiment of the invention, the cationic pectin salt used is calcium pectinate. In the sense of the present invention, pectin is also understood to mean methylated or non-methylated, amidated or non-amidated pectin.

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[Para 49] The galenic forms according to the invention can be administered in all oral forms, in particular in the form of gels and capsules.

[Para 50] These gels and these capsules can be administered simultaneously or successively with other active ingredients, in particular when the gels or the capsules contain enzymes capable of inactivating the antibiotics, they can be administered simultaneously or successively with the preparation of corresponding antibiotics.

[Para 51] The active ingredients administered conjointly with the gels and capsules containing the galenic forms according to the invention are administered orally or via any other method.

[Para 52] The galenic forms according to the invention can be prepared by methods known to the expert or by novel processes which likewise make up part of the invention.

[Para 53] Accordingly, the object of the present invention is also a process for preparation of multiparticular galenic forms, characterised in that an aqueous solution of pectin containing the active ingredient is added dropwise to a concentration of 0.5 to 5% (v/v) in a solution of calcium chloride to form the beads of calcium pectinate, then the beads of calcium pectinate thus obtained are recovered and introduced to an aqueous solution of the polymer cationic.

[Para 54] In an advantageous embodiment of the process, the pectin solution is 4 to 10% (m/v), preferably 4 to 7%, the solution of calcium chloride from 2 to 10% (m/v) and the solution of polymer cationic from 0.5 to 2% (m/v), said solution of polymer cationic preferably being a solution of polyethylenimine.

[Para 55] In an even more advantageous embodiment of the invention the galenic forms are prepared from a solution of pectin at 6% (m/v), a solution of calcium chloride at 6% (m/v) and a solution of polyethylenimine at 1% or at 0.6%.

[Para 56] The beads are maintained in the calcium chloride with slow stirring for 10 min to 1 hour, preferably for 20 min. The reticulation stage by the cationic polymer is performed with slow stirring for 15 to 40 min, preferably for 20 min.

[Para 57] After recovery of the pectinate beads, the beads are dried at a temperature of between 20 and 40°C for 30 min to 10 hours, preferably at 37°C for 2 hours.

[Para 58] The diameter of the particles according to the invention is between 800 and 1500 μm, preferably between 1000 and 1200 μm.

[Para 59] The encapsulation yields are between 50 and 90% or 3-6 UI/beads of β-lactamases, activity expressed in the benzylpenicillin substrate, whether the pectin is amidated or not.

[Para 60] Stability in gastric juice is greater than 10 hours and is likewise very good in intestinal medium USP XXIV, since it is greater than 7 hours (the duration of stability of non-reticulated pectin beads does not exceed 1 hour) and this irrespective of the type of pectin utilised.

[Para 61] Examples 1 to 7 and Figures 1 to 8 which follow illustrate the invention.

[Para 62] Figure 1 shows the effect of reticulation with different concentrations of PEI (0.6; 0.7; 0.8; 0.9 and 1%) on the disaggregation time of amidated pectin beads, placed in three different media: PBS, 0.01 M, pH to 7.4; intestinal medium at pH of 6.8 + 0.1 UPS XXIV; gastric juice at pH of 1.1 USP XXIV.

[Para 63] Figure 2 illustrates the structure of beads containing β-lactamases at the rate of 4.4 UI/bead and reticulated for 20 minutes in PEI at 1% and observed by scanning electron microscopy.

[Para 64] Figure 3 illustrates the release of in vitro β-lactamases from reticulated amidated pectin beads prepared according to Example 1 with PEI concentrations of 0.6 and 0.7% and containing around 5 UI/bead, placed in intestinal medium USP XXIV then in colonic medium (HEPES buffer pH 6 + pectinolytic enzymes).

[Para 65] Figure 4 illustrates the evolution of β-lactamase activity in the stools of mice as a function of time, after oral administration of reticulated pectin beads in PEI prepared according to Example 1 and containing 4.4 UI/bead.

[Para 66] Figure 5 illustrates the structure of beads containing β-lactamases at the rate of 4.4 UI/bead 30 minutes after in vivo administration. The beads are then in the stomach. A and B represent the whole beads and C and D the cut beads.

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[Para 67] Figure 6 illustrates the structure of beads containing β-lactamases at the rate of 4.4 UI/bead 2 hours after in vivo administration. The beads are then in the small intestine. A and B represent the whole beads and C and D the cut beads.

[Para 68] Figure 7 illustrates the structure of beads containing β-lactamases at the rate of 4.4 UI/bead 4 hours after in vivo administration. The beads are then in the colon. A and B represent the whole beads and C and D the cut beads.

[Para 69] Figure 8 illustrates encapsulation, in pectin beads, of free or complex plasmid DNA with cationic lipids (Lipoplexe) or cationic polymer (Polyplexe).

Example 1: Preparation of galenic forms

[Para 70] An aqueous solution of pectin at 6% (OF 400 or OG175C Unipectint by Degussa) was introduced dropwise to a solution of calcium chloride at 6% (m/v). The solution of pectin was introduced to the solution of calcium chloride via Tygon piping connected to a peristaltic pump (Microperpexe LKB Bromma). The solution was passed through a needle of 0.8 mm in diameter (21G, Nedus Terumo) to form drops of pectin which gelled instantly on contact with the calcium chloride (40 ml) and yielded beads of calcium pectinate. The beads were kept in the calcium chloride, with slow stirring, for 20 minutes.

[Para 71] The white beads not containing active ingredient (β-lactamases) were obtained starting out from a solution of amidated (OG 175C) or non-amidated (OF 400) pectin at 6%. For preparation of loaded beads the active ingredient (β-lactamases, penicillinases of type A extracted from Bacillus cereus by Sigma) was mixed in with the solution of pectin in a ratio of 3% (Vpa/Vpectin).

[Para 72] The resulting beads of calcium pectinate were then recovered by filtration, rinsed in distilled water, placed on a Petri dish and dried by kiln at 37°C for 2 hours.

[Para 73] For reticulation in polyethylenimine the undried beads, recovered from the solution of $CaCl_2$ by filtration, were introduced to an aqueous solution of polyethylenimine (PEI) at 1% and were kept there for 20 min with gentle stirring.

[Para 74] The beads prepared from the non-amidated pectin OF 400 contained from 1 to 2.5 UI/beads and the beads prepared from amidated pectin OG175C contained from

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1 to 5 UI/beads.

Example 2: Stability of beads

1. Operational method.

[Para 75] The beads were prepared according to Example 1 with or without the reticulation stage; the duration of reticulation in PEI was 20 minutes in solutions of concentrations ranging from 0.6 to 1%.

[Para 76] The beads were placed either in phosphate buffer (PBS O,01M, pH 7.4), or in media simulating digestive juices (gastric and intestinal USP XXIV) and the disaggregation time was observed.

2. Results.

[Para 77] These are given in Figure 1.

[Para 78] The beads reticulated or not were stable in the PBS and in the gastric medium. However, the non-reticulated beads were unstable in the intestinal medium, whereas the beads according to the invention were stable for over 7 hours.

Example 3: Morphological characteristics of beads.

[Para 79] They are illustrated in Figures 2A to 2D.

[Para 80] The cuts show that the centre of the beads was full and dense. The surface shell corresponds to the PEI. The interior and exterior have different structures.

Example 4: Release kinetics in vitro

[Para 81] 1. Operational method.

[Para 82] Beads reticulated with two different concentrations of PEI (0.6 and 0.7%) were prepared according to Example 1 from amidated pectin and containing 5 UI/bead. They were left for 5 hours in intestinal medium USP XXIV at pH 6.8, then introduced to synthetic colonic medium at pH 6 including pectinolytic enzymes (Pectinex Ultra SPL).

[Para 83] The residual β-lactamase activity in the beads was measured over time by spectrophotometry in the presence of nitrocephine.

2. Results.

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[Para 84] They are illustrated in Figure 3.

[Para 85] After 5 hours of incubation beads in intestinal medium (T_{5H}) less 25% of β-lactamase activity which they contain was released. The release becomes important in colonic medium under the action of pectinolytic enzymes (Tien), for the reticulated beads with 0.6% PEI, while the sample without pectinolytic enzymes (T_{10H} control) had no significant modifications of β-lactamase activity. On the contrary, the beads reticulated with 0.7% PEI did not have their activity diminish after 5H in colonic medium.

[Para 86] Thus the concentration of PEI modifies the resistance of beads and plays on the release time of the active ingredients in colonic medium.

Example 5: Release kinetics in vivo.

[Para 87] 1. Operational method.

[Para 88] This assay was performed on male mice CD1. The beads contain 4 UI/bead.

[Para 89] Gels containing 10 beads were administered per os to the mice. The stools were recovered at time periods of 0, 2H, 3H, 4H, 5H, 6H, 7H and 8H and the dosage of β-lactamases in these stools was realized (assay conducted on 5 animals for each time). In addition, one mouse was sacrificed at times of 30 min, 2H and 4H so as to recover the beads in its digestive tract and observe their morphological modifications by scanning electron microscopy.

2. Results.

[Para 90] These are illustrated in Figures 4 to 7.

[Para 91] The beads arrived intact in the colon after around 3 hours' transit.

[Para 92] The rate of β-lactamases released directly in the stools of mice gathered at different times after absorption of the beads orally shows that the basic β-lactamase activity is low at the outset. Two to 4 hours after administration there was a clear increase in this activity, corresponding to the transit of the beads (Figure 4).

[Para 93] The photos taken by scanning electron microscopy show the integrity of the bead at different places of the digestive tract.

[Para 94] The structure is slightly fragile in the small intestine and the inside was completely destroyed at colonic level where the beads appeared carriers of a cavity.

[Para 95] As illustrated in Figure 5 the particles, having stayed in the stomach, looked very similar to those which had not undergone any treatment (Figure 2). In effect the surface had the same rugged and irregular look (Figures 5A and 5B), owing to the presence of polyethylenimine, and the cross-section of the beads appeared uniform and dense (Figures 5C and 5D).

[Para 96] At the end of 2 h slight deformation of the beads became apparent (Figure 6A), but the particles still had the same surface appearance (Figure 6B) and a dense cross-section (Figure 6C), even though they were made a little fragile by their stay in the small intestine (Figure 6D).

[Para 97] On completion of transit, that is, 4 h after administration, the beads were in the colon; the external appearance of the particles was unchanged (Figure 7A) with the same surface irregularities due to the polyethylenimine (Figure 7B). Yet the cross-section of the beads was hollow (Figures 7C and 7D), due to the fact of degradation of the central network of calcium pectinate by the colonic pectinolytic enzymes. Finally, only the external shell formed by the polyethylenimine remained.

Example 6: Encapsulation of erythromycin esterase.

[Para 98] 6.1 Production of a soluble fraction containing erythromycin esterase

[Para 99] 6.1.1. Operational method

[Para 100] The culture was made from the strain of E. coli C600 pIP1100 from the Pasteur Institute. The culture conditions were the following: inoculation of the Mueller-Hinton medium at 0.5% from a preculture of about 20h, culture volumes of 200 or 400 mL in Erlenmeyer, fixed agitation at 150 rpm, temperature of 37°C.

[Para 101] A GOTS test helped establish that the strain produced much erythromycin esterase.

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[Para 102] 3.6 L of culture of E. coli C600 pIP1100 were concentrated according to the following protocol:

[Para 103] Centrifuging for 15 min at 3400 g

[Para 104] Recovery of cap in potassium phosphate buffer 5mM, pH 7.5, final volume 70 mL

[Para 105] Second centrifuging of supernatant for 15 min at 3400 g

[Para 106] Recovery of cap in 20 mL of potassium phosphate buffer 5mM, pH 7.5

[Para 107] Reuniting of caps of the 2 centrifuges (around 100 mL)

[Para 108] Washing of caps and centrifuging (10 min at 12,000 g)

[Para 109] Second centrifuging of supernatant (10 min 12,000 g)

[Para 110] Final volume of caps recovered in the potassium phosphate buffer: 100 mL.

[Para 111] The erythromycin esterase was an intracellular enzyme. This is why its solubilization required the cells to be broken. This operation was carried out by ultrasonic extraction of centrifuging caps recovered in the potassium phosphate buffer 5mM, pH 7.5 according to the protocol described hereinbelow.

[Para 112] Addition of 1% TritonX100 (v/v)

[Para 113] Cooling to 5°C

[Para 114] Phonolysis 7 cycles of 1 min, initial temperature 5°C, maximal temperature 15°C, power: 100% (500W, 20 kHz) ; temperature taken to 5°C after each cycle

[Para 115] Centrifuging for 10 min at 12,000 g

[Para 116] Recovery of cap in 10 mL of potassium phosphate buffer 5mM, pH 7.5

[Para 117] Recovery and congealing of the supernatant (91 mL) = solution A.

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[Para 118] The erythromycin esterase activity was evaluated by the microbiological dosage in the supernatant and in the insoluble substances (cellular debris) according to techniques known to the expert.

6.1.2. Results

[Para 119] The results are presented in Table 2.

Table 2

Sample	Diameter of inhibition (mm)			
	T0	T30	T60	T120
Supernatant after ultrasound	31	25	18	-
	21	-	18	14
Cap after ultrasound	30	28	24	-
	22.5	-	19.5	19

[Para 120] The erythromycin esterase activity was evaluated from the diameter of inhibition.

[Para 121] The latter was 2 U/mL for the phonolysis supernatant and 1.5 U/mL for the phonolysis cap (1 Unit (U) = 1 μg of erythromycin degraded per min).

[Para 122] The balance of recuperation of the erythromycin esterase activity is presented in Table 3 hereinbelow.

Table 3

Sample	Estimated activity (U/mL)	Volume (mL)	Total estimated activity (U)
Supernatant after ultrasound	2.0	92	184
Cap after ultrasound	1.5	10	15

[Para 123] The results clearly show that the essential element in the erythromycin esterase activity present has been solubilized in the phonolysis medium.

[Para 124] 6.2 Encapsulation of erythromycin esterase

[Para 125] 6.2.1. Operational method

[Para 126] Encapsulation was achieved from the non-purified soluble fraction obtained after breaking the cells (solution A) according to the following protocol.

[Para 127] Solubilization of 0.5 g of pectin in 10 mL solution A to obtain a final concentration of pectin of 5% (solution B). The pectin was added very progressively with magnetic stirring so as not to cause too many abrupt variations in pH. The pH was maintained in the region of 7 by addition of a few drops of soda 1M.

[Para 128] Dispersion of the solution of pectin (solution B) dropwise by means of a peristaltic pump to 40 mL of $CaCl_2$ at 6%. The beads thus formed were kept in the $CaCl_2$ for 20 min, recovered by Büchner filtration then rinsed in demineralised water.

[Para 129] Reticulation of the beads by bath in a solution of PEI at 0.6% for 20 min with magnetic stirring.

[Para 130] Recovery of the reticulated beads by filtration.

[Para 131] The beads were dried at ambient temperature (20°C). 567 beads were prepared in total with 6.1 mL of pectin/solution A mixture, for an activity of 12.2 U.

[Para 132] The dried beads were disaggregated in a buffer HEPES/NaCl/EDTA 1%.

6.2.2. Results

[Para 133] The erythromycin esterase activity present in the initial solution of pectin and that released in the disaggregation medium were dosed according to the same protocol as previously.

[Para 134] The results of the microbiological dosage are presented in Tables 4 and 5.

Table 4

Sample	Average inhibition diameter (mm)
Pectin / Solution A (solution B) –T0	23
Pectin / Solution A –T3h	19
Disaggregated beads – T0	24
Disaggregated beads – T3h	18

Table 5

Sample	Estimated activity
Pectin (Solution B)	2.4
Disaggregated beads	2.2

[Para 135] The results show that the activity measured in the presence of pectin (solution B) is 2.4 U, while the theoretical activity present should be around 12 U (6.1 mL at 2 U/mL, according to the dosage of erythromycin esterase in the phonolysis supernatant (Table 3).

[Para 136] The dosage of enzymatic activity of beads after disaggregation had been estimated at 2.2 U; it representsed 90% of the initial activity introduced to the beads.

[Para 137] These results help confirm unambiguously the presence of erythromycin esterase activity in the final fraction after encapsulation of the enzyme and disaggregation of the beads.

Example 7: Encapsulation of DNA in the calcium pectinate beads

[Para 138] 7.1 Preparation of DNA

[Para 139] The active ingredient encapsulated here was a plasmid radiomarked with Phosphore 33. The radiomarking was done by means of the Nick Translation Kit N5500 from Amersham Biosciences according to the protocol described by the supplier.

[Para 140] 7.2 Encapsulation

[Para 141] 7.2.1. Operational method

[Para 142] The encapsulated DNA was either in free form, or complexed with cationic lipids (Lipoplexe) or a cationic polymer (Polyplexe) according to the operational method described in Example 1.

[Para 143] For free DNA, around 5 μg of DNA radiomarked in solution in 750 μL of MilliQ water were introduced to 0.75g of a pectin solution, amidated or not, at 10% so as to obtain a final concentration of pectin of 5%. In the case of the lipoplexes, 375 μL of an aqueous solution of radiomarked DNA were mixed with 375 μL of a suspension of cationic liposomes (N/P ratio of 10). The 750 μL of resulting lipoplexes were then mixed with 0.75 g of solution of pectin at 10% so as to obtain a final concentration of pectin of 5%.

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[Para 144]   In the case of polyplexes, 375 μL of an aqueous solution of radiomarked DNA was mixed with 375 μL of an aqueous solution of PEI 4mM. 375 μL of the suspension of polyplexes thus obtained were then mixed with 0.75 g of pectin solution at 10% to provide a final concentration of pectin of 5%.

[Para 145]   The beads of calcium pectinate encapsulating the free or complex DNA were then prepared from solutions obtained hereinabove according to the method described in Example 1.

[Para 146]   The concentration of calcium chloride utilized here was 5% and that of PEI for reticulation was 0.6%.

7.2.2. Results

[Para 147]   They are illustrated in Figure 8 which shows the encapsulation yields of a plasmid DNA in amidated or non-amidated pectin beads.

[Para 148]   The encapsulated DNA was either in free form, or complexed in cationic lipids (Lipoplexe) or a cationic polymer (Polyplexe).

[Para 149]   The encapsulation yields of DNA varied between 60 and 90% according to the type of pectin used. They were generally more significant with amidated pectin. Complexing with lipids or a cationic polymer did not cause significant modifications to these yields, which remained relatively high.